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Review

The Conventional and Breakthrough Tool for the Study of L-Glutamate Transporters

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Abstract: In our recent report, we clarified the direct interaction between excitatory amino acid transporter (EAAT) 1/2 and polyunsaturated fatty acids (PUFAs) by applying electrophysiological and molecular biological techniques to Xenopus oocytes. Xenopus oocytes have a long history of use in the scientific field, but they are still attractive experimental systems for neuropharmacological studies. We will therefore summarize the pharmacological significance, advantages, (especially in the study of EAAT2), and experimental techniques that can be applied to Xenopus oocytes; our new findings concerning L-glutamate (L-Glu) transporters and PUFAs; and the significant outcomes of our data. The data obtained from electrophysiological and molecular biological studies of Xenopus oocytes have provided us with further important questions, such as whether or not some PUFAs can modulate EAATs as allosteric modulators and to what extent docosahexaenoic acid (DHA) affects neurotransmission and thereby affects brain functions. By combining Xenopus oocyte experiments and more translational approaches, we can clarify the functions of proteins that are difficult to examine using cultured cells and the physiological roles of these proteins in brain functions. These approaches can lead to the development of the new central nervous system (CNS) drugs targeting molecules and investigations of their effectiveness.

Keywords: Xenopus oocyte; glutamate transporter; EAAT2; two-electrode voltage clamp (TEVC); overexpression; excitotoxicity

1. Basic background on EAAT2 and other L-glutamate transporters in the central nervous system

Human excitatory amino acid transporters (EAATs) have 5 subtypes, EAAT1, 2, 3, 4, and 5 (EAAT1-3 [1], EAAT3 [2], EAAT4 [3], EAAT5 [4]). EAAT2 is the predominant excitatory neurotransmitter transporter that accounts for approximately 90% of L-glutamate (L-Glu) uptake in the forebrain [5], meaning that EAAT2 is essential for healthy brain functioning. EAAT2 protects neurons from excitotoxicity, the neuron-specific cell death mechanism caused by prolonged elevation of extracellular L-Glu [6], and refines synaptic transmission. EAAT2 is expressed mainly in astrocytes, but 5 to 10% of total EAAT2 expression is found in neurons [7]. The extracellular and intracellular electrochemical gradient of Na^+ is a driving force of transport. The principle of EAAT2 transport is as follows: 1 glutamate transport is coupled to the influx of 3 Na^+ and 1 H^+ followed by the outflux of 1 K^+, resulting in electrogenicity [8] (Figure 1A). This coupling maintains a transmembrane L-Glu concentration gradient ($[Glu]_{in}/[Glu]_{out}$) exceeding 10^6-fold under physiological conditions [8], i.e., the $[Glu]_{in}$ of astrocytes is approximately 2–3 mM L-Glu in their cytoplasm, and $[Glu]_{out}$ under resting-state conditions is approximately 25 nM [9]. Transport against the concentration gradient uses ATP derived from the Na^+/K^+-ATPase pathway [10].

2. EAAT2 pharmacology

As described above, because EAAT2 is the predominant L-Glu transporter in the frontal cortex, defective EAAT2 transport leads to excitotoxic neuronal death [11–14], which has been identified as one of the pathological changes in Huntington's disease [14,15], amyotrophic lateral sclerosis (ALS) [12,16], and schizophrenia [13]. EAAT2 has, therefore, become a therapeutic target in central nervous system drug development [17,18].

Despite the large demands for EAAT2-selective activators, they have not advanced beyond the clinical trial phase [18–21]. In preclinical research fields, various kinds of EAAT2 modulators are used (Table 1). These modulators are categorized into three classes: (1) substrate-type (competitive) inhibitors, (2) nonsubstrate-type blocker-type (competitive) inhibitors, and (3) allosteric-type (noncompetitive) enhancers. (1) and (2) were developed based on the structures of the L-Glu analogue (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid [AMPA], kainic acid, etc.) as well as substrates such as L-Glu and L-Aspartate (L-Asp) and inhibited EAAT-meditated transport activity through binding to the orthosteric site. Because orthosteric sites are well conserved among EAATs [22], substrate-type inhibitors interact with multiple EAATs to some extent. Furthermore, some substrate-type inhibitors are transported in the same manner as the real substrate. As a result, they cause the reduction of substrate uptake without any effects on the ion flux, i.e., the transport currents of EAATs (see the following Chapter 3-2). Table 1 shows widely used EAAT modulators in EAAT2 studies, which are categorized as (2) and (3). D,L-threo-β-benzyloxyaspartate (TBOA) [23,24] and TFB-TBOA [25] are broad EAATs blockers. N(4)-[4-(2-bromo-4,5-difluorophenoxy)phenyl]-L-asparagine (WAY213613) [26] and dihydrokainic acid (DHK) [1] are EAAT2-selective blockers. Parawixin1 [27,28] and 3-((4-cyclohexylpiperazin-1-yl)(1-phenethyl-1H-tetrazol-5-yl)methyl)-6-methoxyquinolin-2(1H)-one (GTG949) [29] are allosteric-type EAAT2-selective enhancers. Parawixin1 [27,28] and GTG949 [29] are allosteric-type enhancers (3) and selectively enhance EAAT2-mediated transport activity through binding to an allosteric site, namely, a site other than the substrate binding site. Parawixin1 is the venom extract of the spider *Parawixia bistriata*, and its exact structure has not been clarified. GTG949 was developed by virtual screening by adding functional groups to parawixin1. In addition to these direct modulators, ceftriaxone, a β-lactam antibiotic, enhances transport activity by increasing EAAT2 expression levels. Ceftriaxone increases EAAT2 activity in rodent brains through an increase in EAAT2 expression levels [30] and is expected to be a breakthrough in the treatment of ALS. Despite promising preclinical data, ceftriaxone could not reveal the significant effects in phase III clinical trials [31].

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Table 1. Pharmacological profiles of EAAT2 inhibitors and enhancers.

Category	Compounds	Activity	Structural origin	Major info	Refs
(2)	**TBOA** DL-threo-B-benzyloxyaspartate	Broad, competitive blocker	Aspartate	• IC_{50}: EAAT1 = 70 μM, EAAT2 = 6 μM, EAAT3 = 6 μM, EAAT4= 4.4 μM, EAAT5=3.2 μM	23, 24
	TFB-TBOA (2S,3S)-3-{3-[4-(trifluoromethyl)benzoylamino]benzyloxy}aspartate	Broad, competitive, long lasting, blocker	Aspartate	• IC_{50}: EAAT1 = 22 nM, EAAT2 = 17 nM, EAAT3 = 300 nM • Relatively weak affinity toward NMDA receptors (IC_{50} = 49 μM)	25
	WAY213613 N(4)-[4-(2-bromo-4,5-difluorophenoxy)phenyl]-L-asparagine	Potent EAAT2 competitive blocker	Aspartate	• IC_{50}: EAAT1 = 5 μM, EAAT2 = 85 nM, EAAT3 = 3 μM	26
	DHK Dihydrokainic acid	Selective EAAT2, competitive blocker	Kainate	• IC_{50}: EAAT1 > 3 mM, EAAT2 = 23 μM, EAAT3 > 3 mM • Agonist for kainite glutamate receptor (IC_{50}=6 μM)	1, 23
(3)	**Parawixin1**	Selective EAAT2 allosteric enhancer		• The venom extract of the spider *Parawixia bistriata* • Enhanced glutamate uptake in cortical synaptosomes and protected neurons from excitotoxic death in a rodent model of induced ischemia	27, 28
	GT949 3-((4-cyclohexylpiperazin-1-yl)(1-phenethyl-1H-tetrazol-5-yl)methyl)-6-methoxyquinolin-2(1H)-one	Selective EAAT2 allosteric enhancer		• Developed by the virtual screening by adding to some functional groups on the parawixin1 • EC_{50}: EAAT2= 0.26 nM • Exhibits no significant effect on dopamine transporter, serotonin transporter and norepinephrine transporter or NMDA receptors	29

3. Reasons to choose Xenopus oocytes for the study of EAAT2

Cells used for the pharmacological and physiological study of EAAT2 should meet the following two requirements: the cells should express enough EAAT2, and the molecular functions of the cells can be quantitatively investigated. Xenopus oocytes meet these two requirements and are appropriate for the study of EAAT2. We will explain this in the following paragraphs.

3.1. Advantages of Xenopus oocytes that overexpress EAAT2

EAAT1 and EAAT2 are mainly expressed in astrocytes [32,33], while EAAT2 is also expressed in neurons, but to a lesser extent. However, in the primary culture of astrocytes, the expression level of EAAT2 is very low [11,34], and the functional predominance switches from EAAT2 to EAAT1 [35–38]. In vitro substrate uptake assays are therefore performed using cell lines such as the Human Embryonic Kidney 293 (HEK 293) and African green monkey kidney fibroblast-like (COS) cell lines, in which heterologous expression of cloned transporters has been induced. The problems in these in vitro assays are unignorable variations in the expression levels among cells as well as experimental batches. In contrast, Xenopus oocytes are capable of expressing high amounts of the targeted proteins, and the expression levels are so stable that statistical analyses among experiments are possible. The following are the additional technical advantages of the use of Xenopus oocytes:

■ Approximately 200 or more oocytes at defolliculated stage V or VI that are suitable for cRNA injection can be collected from one Xenopus. Four to six operations with 1-week intervals are possible per Xenopus. Additionally, 3 injections are possible for the oocytes collected in one operation. Therefore, ready-to-use oocytes can be obtained 12 times a month.

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■ The transfection protocol is easy and feasible. To overexpress the targeted protein in Xenopus oocytes, 50 nl of capped cRNA (10 ng) solution is directly injected into an oocyte by a nanoinjector installed with a glass microelectrode. An oocyte is a spherical cell with a diameter of 1–1.2 mm, large enough to confirm successful injection by checking the swelling of the oocyte. Furthermore, the diameter of the microelectrode tip is 20-25 μm, which enables the minimization of membrane damage and the initiation of electrophysiological recording the next day.

■ By modulating the interval between the injection and the analysis, the expression level of the targeted proteins can be regulated. Conversely, experiments using oocytes expressing almost the same level of the targeted proteins can be performed.

■ The interactions of the targeted protein with the accessory subunits and/or modulatory proteins can be examined through the cotransfection of these proteins at the optimal ratio [e.g., protein interacting with C kinase 1 (PICK1), which containing postsynaptic density protein (PDZ) domain, with GLT1 (EAAT2 in rodents) [39]].

3.2. Measurement of the activity of EAAT2 expressed in Xenopus oocytes

The quantification of the amount of radioisotope (RI)-labelled substrates transported into the oocytes is a conventionally and widely used protocol for the quantification of EAAT2 activity [19,40]. Because the transport activity depends on the membrane potential, accurate quantification should be performed under the membrane potential clamp [41]. As described in Chapter 1, substrate transport is coupled to the influx of 3 Na^+ and 1 H^+ followed by the outflux of 1 K^+, resulting in L-Glu transport-coupled currents (I_{AA}) [8]. Furthermore, the uncoupled Cl^- anion channel is opened (I_{Cl}) by substrate binding. The model by Wadiche shows that the total L-Glu-induced EAAT currents (I_{total}) are the sum of I_{AA} and I_{Cl} [41] (Figure 1B-1). Electrophysiological techniques allow real-time detection of the effects of compounds on L-Glu transport. Furthermore, these techniques can also detect heteroexchange associated with transport, which is impossible to detect by RI labeled substrate assay. The current-voltage (IV) relationships of I_{AA} show inward rectification with no reversal potential, that is the membrane potential at which the direction of ionic current reverses, while those of I_{Cl} show linear with reversal potential and outward current at positive membrane potentials. In I_{total}, the outward current is due to I_{Cl} and the reversal potential is dependent on the relative magnitude of I_{AA} and I_{Cl} (Figure 1B-3). The amplitude of I_{total} is dependent on the amount of transported of substrates (Figure 1B-2), while the reversal potential of I_{total} is independent of the amount of substrates transported (Figure 1B-2). EAAT1-5 differ in the proportion of I_{AA} and I_{Cl} in I_{total}. The neuronal transporters EAAT4 and EAAT5 act primarily as Cl^- anion channels due to I_{Cl} dominance [3,4]. On the other hand, EAAT1, EAAT2 and EAAT3 have smaller I_{Cl} than I_{AA}. Specifically, I_{AA} is predominant, and the contribution of the Cl^- anion current is very small in EAAT2 I_{total} [41]. Two-electrode whole-cell voltage clamp (TEVC) methods show that the IV relationships for the L-Glu-induced EAAT2 current have no reversal potential up to +60 mV and a very similar curve to I_{AA} in Xenopus oocytes overexpressing EAAT2 (Figure 1C), meaning that EAAT2 transport activity can be quantified as substrate-induced EAAT2 currents. In line with this, the open probability of Cl^- anion channels of EAAT2 transfected in HEK293 cells was confirmed to be 0.06±0.01% [42]. The following are the additional advantages of electrophysiological recording in Xenopus oocytes.

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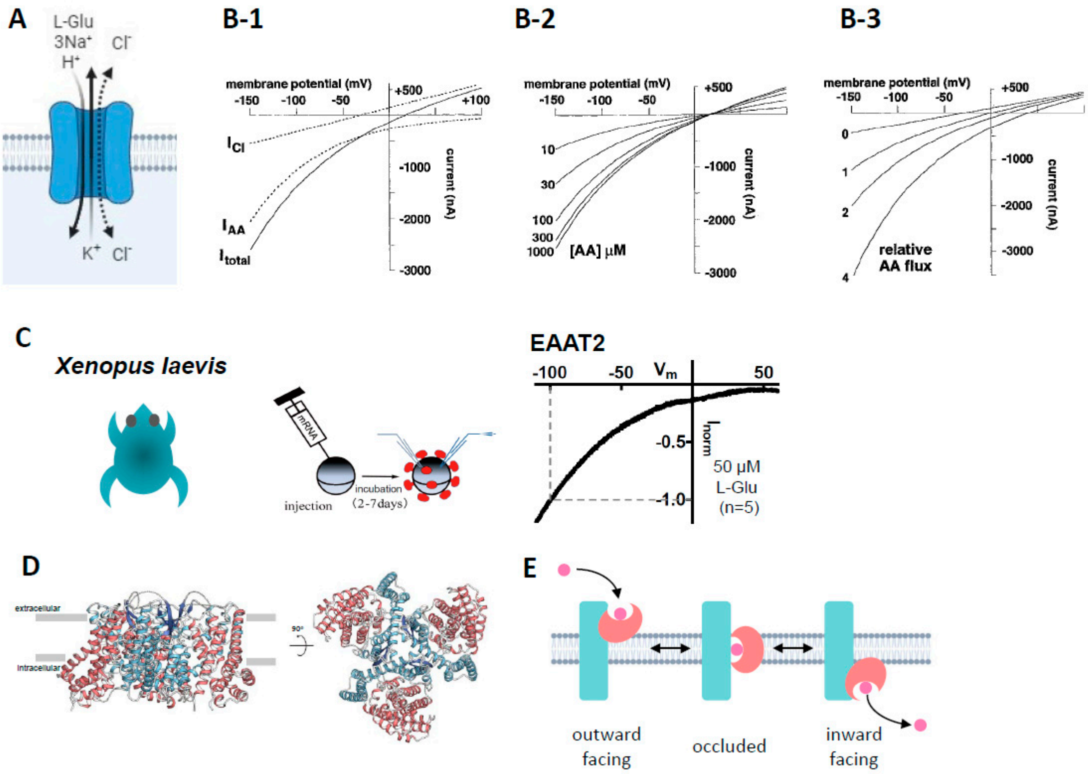

Figure 1. (A) Substrate and coupling ions of transport for EAATs. Substrate, such as L-Glu, L-Asp or D-Asp, transport through EAATs is coupled to the cotransport of 3 Na+ and 1 H+ followed by the counter transport of 1 K+. In addition, the binding of substrates and Na+ to EAATs activates uncoupled Cl- anion currents. (B) B-1 Model of total transporter current (solid line). The total L-Glu-induced EAAT currents (solid line: Itotal), electrophysiologically recorded using TEVC methods from EAAT-expressing Xenopus oocytes, represent the sum of the coupled L-Glu transport currents (dotted line: IAA) and the uncoupled Cl- anion currents (dotted line: ICl). B-2 The predicted reversal potential of the net current (Itotal) is independent of substrate concentration when the concentration dependence of IAA and ICl is the same. However, the amplitude of Itotal is dependent on the substrate concentration. B-3 The absolute reversal potential of Itotal is dependent on IAA relative to that of ICl. [From Wadiche et al. [41] @ 2023, with permission from Elsevier] (C) Oocytes were collected from anaesthetized Xenopus laevis. The isolated oocytes were then treated with collagenase (2 mg mL−1, type 1), and capped mRNA was injected into either defolliculated stage V or VI oocytes. The oocytes were incubated for 2–7 d at 18 °C in ND96 solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES (pH 7.5) supplemented with 0.01% gentamycin. TEVC recordings from the oocytes were performed at room temperature (25 °C) using glass microelectrodes filled with 3 M KCl (resistance = 1–4 MΩ) and an Ag/AgCl pellet electrode. IV relationship for L-Glu (50 μM)-induced EAAT2 current. To examine the IV relationship, the L-Glu–induced current was calculated through the subtraction of the steady-state current from the L-Glu-induced current. The curves were obtained with a holding potential of −60 mV applying an 8000 ms ramp pulse from −110 to +60 mV. Data are shown as the values normalized to that obtained with 50 μM L-Glu at -100 mV. Means, n=5. (D) Overall structure of human EAAT2 as viewed from the membrane plane (left) and the intracellular side (right). The trimerization domain is in blue, and the transport domain is in red. [From Kato et al. [60]] The protomer of EAAT2 is divided into two distinct functional components: one is a rigid scaffold domain that mediates interprotomer interactions and is located in the centre of the trimer, and the other is a transport domain containing the substrate-binding site [65]. (E) Elevator motion.

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Schematic representation of the transport cycle of EAATs. The transport domain (red) moves across the membrane relative to the trimerization domain (blue). The transported L-Glu in pink.

■ Because a Xenopus oocyte is a large single cell, TEVC methods can be applied. Among the various types of electrophysiological techniques, it is rather easy to become proficient in performing TEVC. Furthermore, the oocyte membrane is so strong and stable that obtaining recordings from 10-30 oocytes per day and for longer than 30 min per oocyte is possible [39,43,44].

■ Because the current values are so large (31.4±21.7 nA, n=27, Holding potential=- 50 mV) in Xenopus oocytes transfected with EAAT2 [45], accurate quantification of any modulation is possible.

■ It is easy to modify intracellular and/or extracellular conditions during recording. For example, it is possible to change intracellular conditions by filling glass microelectrodes with compounds such as H_2O_2 or DTT [44]. It is also possible to change the extracellular conditions by changing the pH, Cl^- concentration, etc. Furthermore, through incubation with conditioned medium for ~2 days, the chronic effects of compounds can also be examined [46].

■ In addition to whole oocyte clamp, more detailed experiments, such as single channel recording, outside-out patch clamp, and inside-out patch clamp, can be performed [47,48].

3.3. Application of molecular biological techniques to Xenopus oocytes

To investigate the structural and functional properties of the targeted functional proteins in detail, site-directed mutagenesis, i.e., cysteine substitution, chimaera, etc., can be applied. The substituted cysteine accessibility method (SCAM) is typically used to investigate the structural information of membrane proteins [49,50]. In SCAM, local steric and electrostatic environments are probed by sulfhydryl reactive reagents because of the reactiveness of the substituted cysteines. This method has been applied to clarify the modulator binding sites and the regions related to the different functional states of many kinds of membrane proteins. The membrane topologies of EAATs have been studied by this method as well [51,52]. In addition, by inserting some pairs of cysteines and assaying their ability to form disulfide bonds, the proximity and mobility relationships between specific positions within transporters have been clarified [53–55]. In Greek mythology, a chimaera is a monster that is a fusion of a lion, goat, and dragon. In molecular biology, a chimaera refers to cells or animals in which two or more genotypes are fused. In terms of the study of EAATs, quite a few chimaera combinations have been made to identify the binding sites for substrates and ions [56,57]. This technique was used to define the functional domains related to transport blockers. Vandenberg's group defined the combination of helical hairpin (HP) 1, HP2, transmembrane (TM) 7 and TM8 to determine the sensitivity to transport blockers such as kainic acid [56] and 3-methylglutamate [57] (see Figure 2D about the membrane topology of EAATs).

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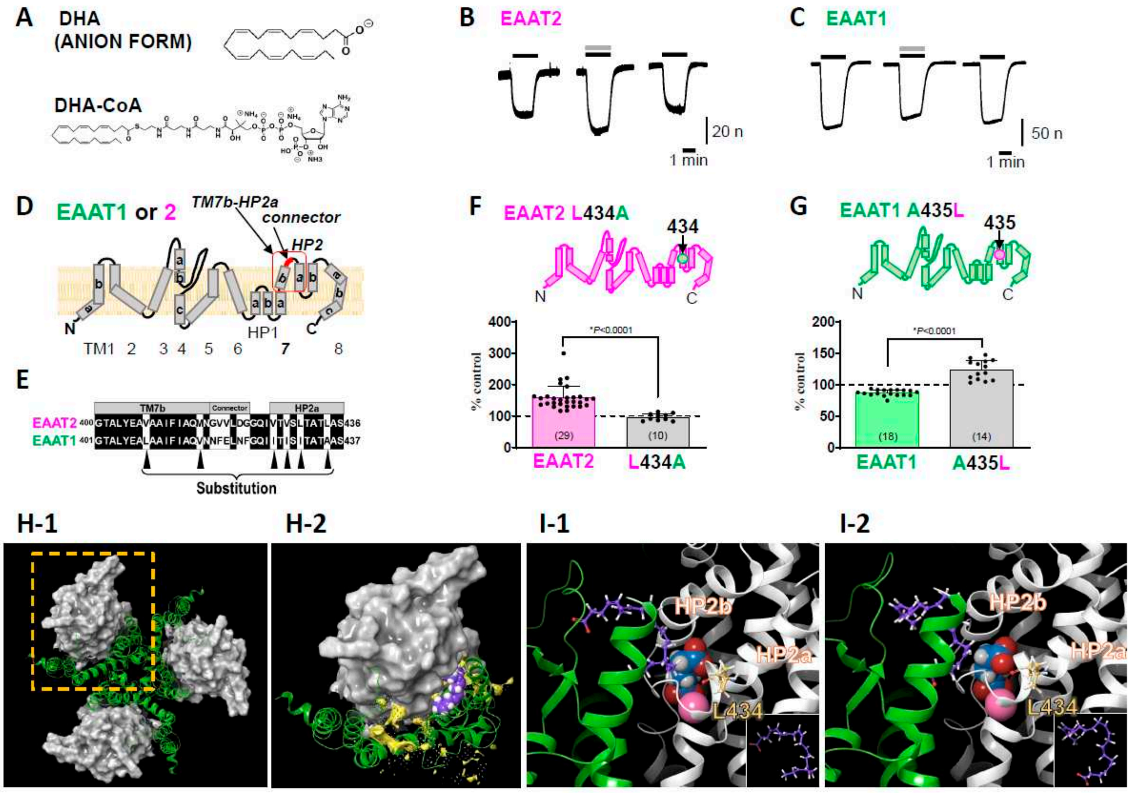

Figure 2. (A) The structure of DHA and DHA-CoA. (B) Representative traces of L-glutamate (L-Glu, 50 μM for 2 min, black bar)-induced current obtained from Xenopus oocytes overexpressing EAAT2 clamped at −50 mV in the absence or presence of DHA (100 μM for 2 min, grey bar). When coapplied, DHA increased the L-Glu-induced EAAT2 current, and the effect disappeared after washout. (C) Representative traces of L-Glu-induced EAAT1 currents in the absence or presence of DHA. When the compounds were coapplied, DHA tended to decrease the EAAT1 current, and the effect disappeared after washout. (D) Topology of EAAT1 or EAAT2. EAAT2 is organized into eight transmembrane (TM1-8) and two helical hairpins (HP1 and HP2), which are re-entrant loops. TM7b-HP2a sequence and connector sequence in the red square. (E) Amino acid alignment from TM7b to HP2a of EAAT2 and EAAT1. The common amino acids are shown on a black background. Single amino acid back-mutations were performed at the sites indicated by black arrowheads in the EAAT1 (EAAT2 TM7b-HP2a) chimaera. (F) Top. Topology of EAAT2 L434A. Bottom. Comparison of the effects of DHA on EAAT2 and EAAT2 L434A. Data are shown as rates of increase by DHA. (G) Top. Topology of EAAT1 A435L. Bottom. Comparison of the effects of DHA on EAAT1 and EAAT1 A435L. Data are shown as rates of increase by DHA. (H) Proposed binding conformation for DHA in the transport domain/scaffold domain interface of the EAAT2 homology model in the outward facing state (OFS). H-1 A a1. Extracellular view of the trimerized EAAT2 OFS homology model based on the EAAT1 crystal structure. The scaffold domain is shown as a green ribbon. The transport domain is shown as a grey surface. H-2 Magnified monomer in the hatched square in H-1 in the presence of DHA. The lipid crevice calculated by SiteMap exists at the interface between the scaffold domain and the transport domain (yellow space). DHA is docked to the lipid crevice (carbon: purple spheres; hydrogen: white spheres) (I) Docking poses of DHA in the lipid pocket in the vicinity of HP2 according to the induced fit docking protocol. The scaffold domain and transport domain are shown in green and grey ribbons, respectively. The carbons in DHA and EAAT2 L434 are represented by purple and yellow sticks, respectively. The atoms in L-Glu are shown as follows: carbon, blue sphere; hydrogen, white sphere; oxygen, red sphere; nitrogen, hidden. Na+ is shown as a pink sphere. Two types of DHA conformations could be visualized according to the position of the carboxylic group, i.e., one with a carboxyl group on the upper side (I-1) and the other with a carboxyl group on the lower side (I-2). Both of them have similar U-shaped conformations. The inset shows the DHA conformations in each case. The three-dimensional position of DHA is in close proximity to the L-Glu

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binding site and Na+ binding site. Error bars represent the mean ± SD. The numbers written within parentheses in each figure represent the number of independent experiments. Statistical differences between groups were determined by two-tailed paired Student's t test. P values are indicated in each figure panel. [From Takahashi et al. [45]].

4. New findings about the interactions between PUFAs and EAAT2 obtained with Xenopus oocyte experiments

In this section, we will present our recent work regarding the modulation of EAAT2 function by docosahexaenoic acid (DHA) [45], in which electrophysiological and molecular biological techniques were applied to Xenopus oocytes overexpressing EAAT2. Because EAAT1 (GLAST in rodents) is also expressed in astrocytes (especially in the cerebellum) [58], we examined the effects of DHA (C22:6) (Figure 2A) and other polyunsaturated fatty acids (PUFAs) on EAAT2 compared with EAAT1. DHA has long been known to enhance synaptic transmission through multiple mechanisms, and L-Glu transporters are candidate proteins involved in target molecules.

We found that DHA significantly increased L-Glu-induced EAAT2 currents (Figure 2B) but slightly decreased L-Glu-induced EAAT1 currents (Figure 2C) using TEVC in Xenopus oocytes transfected with EAAT1 or EAAT2. Although there are some reports indicating the effects of DHA on EAATs, whether DHA interacts with EAATs directly has yet to be elucidated. To solve this problem, we attempted to identify the key amino acid for the DHA-EAAT2 interaction by EAAT1/2 chimaeras, focusing on the differences in the effects of DHA on EAAT1 and EAAT2. EAAT2 functions as a homotrimer, with each protomer having eight TM sites (TM1-8) and two HPs (HP1 and HP2), which are re-entrant loops (Figure 2D). The protomer is divided into two distinct functional components: one is a rigid scaffold domain (TM1, 2, 4 and 5) that mediates interprotomer interactions and is located in the centre of the trimer, and the other is a transport domain (TM3, 6, 7, 8, HP1 and HP2) containing the substrate-binding site [59–61] (Figure 1D, 2H). When we examined the effect of DHA-coenzyme A (DHA-CoA), a membrane-impermeable DHA analogue (Figure 2A), DHA-CoA increased the EAAT2 current almost to the same extent as DHA, suggesting that DHA approaches EAAT2 from the outside of cells. Therefore, we focused on the TM7b-HP2a sequence, the extracellular region of the transport domain (red square in Figure 2D). We first constructed EAAT2-based EAAT1/2 chimaeras. However, as sometimes occurs in chimaera experiments, the EAAT2-based chimaera for which its TM7b-HP2a was substituted to that of EAAT1 was nonfunctional. We therefore changed the chimaera from the EAAT2-based chimaera to the EAAT1-based chimaera. When the EAAT1 TM7b-HP2a region was replaced by that of EAAT2 (EAAT1[EAAT2 TM7b-HP2a]), the effect of DHA on the L-Glu current was enhanced. We first suspected that the least conserved region in TM7b-HP2a, the "connector" in Figure 2D, could cause the differences in responses between EAAT1 and EAAT2. However, when we replaced the "connecter" sequence of EAAT1 with that of EAAT2, i.e., in EAAT1 (EAAT2 connecter), the augmentative effect of DHA was not induced. We therefore focused on the amino acids in TM7b-HP2 other than the "connector". Six amino acids (Val407, Met415, Val426, Val428, Leu430, and Leu434 in EAAT2) in TM7b-HP2a are different between EAAT1 and EAAT2 (black arrowheads in Figure 2E), so we back-mutated the above six amino acids one by one to the original EAAT1 amino acid. Among the mutants, only L434A showed a complete disappearance of the effect of DHA. We confirmed that a single mutation of Leu434 in WT EAAT2 to Ala completely suppressed the effect of DHA (Figure 2F). Furthermore, a single mutation of Ala435 in WT EAAT1 (corresponding to EAAT2 Leu434) to Leu also changed the effect from inhibition to enhancement (Figure 2G). The data above strongly suggest the direct interaction of DHA with EAAT2.

To confirm the feasibility of our hypothesis, we next performed a docking simulation between DHA and EAAT2. The dynamics of the scaffold domain and transport domain are involved in the substrate transport processes of L-Glu transporters. The first transport concept was developed by Jardetzky as an alternating access model in 1966 [62]: outward facing state (OFS), occluded state, and inward facing state (IFS). The structural basis for transporter function is the elevator movement (Figure 1E) of the scaffold domain/transport domain backed up by the four crystal structures of Glt_{Ph}

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[OFS: [59,63] →intermediate (i) OFS: [64] →unlocked IFS: [65] →IFS: [66]]. We performed a docking analysis of DHA and EAAT2 OFS using EAAT1 as a template (protein data bank ID [PDBID]: 5LLM) [67]. Among 7 candidate combinations of docking sites and DHA poses, which were proposed by a standard induced fit docking protocol [68–70], the two top-scoring combinations are shown in Figure 2H-I. By performing calculations with SiteMap (SiteMap, Schrödinger, LLC), we found that DHA was docked in the lipid crevice, facing the interface of the transport domain/scaffold domain, which is close to the binding sites of the substrate and Na^+. The DHA poses are U-shaped in both cases (Figure 2I) [45].

5. New insights suggested by the interactions between DHA and L-Glu transporters

5.1. Some PUFAs modulate EAATs as allosteric modulators

Docking analysis of EAAT2 and DHA suggests that the most stable docking site is located at the transport domain/scaffold domain interface close to the binding sites of the substrates and Na^+. Some allosteric modulators, such as GTG949, an EAAT2 selective enhancer [29], and UCPH101, an EAAT1 selective blocker [67,71], interact with the transport domain/scaffold domain interface and affect the movement of the transport domain, namely, elevator motion [67]. Detailed structural studies on Glt_{Ph}, an archaeal EAAT homologue of the thermophilic prokaryote *Pyrococcus horikoshii,* provide supportive evidence for the direct interactions between transporters and lipids, including PUFAs [63,65,72]. In addition, the crystal structure analysis of Glt_{Ph} in the unlocked IFS suggests that the binding of lipids to the interface may facilitate the sliding of the transport domain. Most recently, cryo-electron microscopy (cryoEM) analysis has provided evidence for the direct interactions of EAAT2 and lipids [61]. Our data and recent related findings suggest that DHA allosterically regulates EAAT2 by enhancing the sliding of the transport domain. Furthermore, we also clarified that among 10 fatty acids [docosapentaenoic acid (DPA, C22:5), docosatetraenoic acid (C22:4), docosatrienoic acid (C22:3), eicosapentaenoic acid (EPA, C20:5), arachidonic acid, (ARA, C20:4), eicosatrienoic acid (C20:3), eicosadienoic acid (C20:2), α-linolenic acid (ALA, C18:3), linoleic acid (C18:2) and oleic acid (C18:1)], only DPA, EPA, ARA and ALA showed augmentative effects on EAAT2 and slight inhibition of EAAT1 through the same mechanisms that require interactions with Leu434. Our data further suggest that a particular three-dimensional structure of PUFAs leads to the allosteric modulation of EAAT2.

5.2. Physiological significance: The potential of DHA as a neurotransmission modulator

The interactions between PUFAs and synaptic transmission were previously reported for ARA (w-6, C20:4) [73–75]. However, there remain many PUFAs whose effects on synaptic transmission are unknown. DHA is the most abundant w-3 PUFA in the mammalian brain [76–78] and the major constituent of membrane phospholipids [79]. DHA is synthesized in astrocytes from precursor fatty acids [80–82] and supplied to neuronal membranes as well. Under stable conditions, DHA is esterified to phosphatidylserine or phosphatidylethanolamine. When L-Glu stimulates neurons and astrocytes via L-Glu receptors [83,84], phospholipase A2 (iPLA2) is activated, thereby triggering DHA release [85,86]. The previous data and our findings raise the possibility that endogenous DHA modulates excitatory synaptic transmission. Some studies have reported favourable roles for DHA in human cognition and behaviour; however, consistent conclusions have not been obtained [87]. In animal models and in vitro models, supporting data have been shown. EAATs control synaptic transmission, neurotransmitter spillover, and long-term plasticity [88–91]. Furthermore, DHA facilitates corticostriatal long-term potentiation (LTP), and selective inhibition of iPLA2 abolishes the expression of LTP [92], which is reversed by DHA [93]. More translational studies are needed to determine the effects of DHA on brain functions.

6. Summary

We could clarify the direct interaction between EAAT1/2 and DHA by applying electrophysiological and molecular biological techniques to Xenopus oocytes. These fundamental data are important in terms of raising the next questions, such as whether or not some PUFAs can modulate EAATs as allosteric modulators and to what extent DHA affects neurotransmission and thereby affects brain functions. To address these questions, more cross-sectional and translational approaches are needed. In summary, by combining Xenopus oocyte experiments and more translational approaches, we can clarify the functions of proteins that are difficult to examine using cultured cells and their physiological roles in brain functions. These approaches can lead to the discovery of new targets and the development of new drugs.

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Abbreviations

ALS	amyotrophic lateral sclerosis
DHA	docosahexaenoic acid
EAAT	excitatory amino acid transporter
HP	helical hairpin
IFS	inward facing state
iPLA2	Ca^{2+}-independent phospholipase A2
IV relation	current-voltage relation
L-Asp	aspartate
L-Glu	glutamate
OFS	outward facing state
PUFA	polyunsaturated fatty acid
SCAM	substituted cysteine accessibility method
TEVC	two-electrode whole cell voltage clamp
TM	transmembrane

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